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Effect of sulfur availability on the integrity of amino acid biosynthesis in plants

Review Article

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Summary. Amino acid levels in plants are regulated by a complex interplay of regulatory circuits at the level of enzyme activities and gene expression. Despite the diversity of precursors involved in amino acid biosynthesis as providing the carbon backbones, the amino groups and, for the amino acids methionine and cysteine, the sulfhydryl group and despite the involvement of amino acids as substrates in various downstream metabolic processes, the plant usually manages to provide relatively constant levels of all amino acids. Here we collate data on how amino acid homeostasis is shifted upon depletion of one of the major biosynthetic constituents, i.e., sulfur. *Arabidopsis thaliana* seedlings exposed to sulfate starvation respond with a set of adaptation processes to achieve a new balance of amino acid metabolism. First, metabolites containing reduced sulfur (cysteine, glutathione, S-adenosylmethionine) are reduced leading to a number of downstream effects. Second, the relative excess accumulation of N over S triggers processes to dump nitrogen in asparagine, glutamine and further N-rich compounds like ureides. Third, the depletion of glutathione affects the redox and stress response system of the glutathione-ascorbate cycle. Thus, biosynthesis of aromatic compounds is triggered to compensate for this loss, leading to an increased flux and accumulation of aromatic amino acids, especially tryptophan. Despite sulfate starvation, the homeostasis is kept, though shifted to a new state. This adaptation process keeps the plant viable even under an adverse nutritional status.

Keywords: Sulfur – Nitrogen – Transcriptomics – Metabolomics

Introduction

For land plants, sulfur is an indispensable inorganic nutrient ranking in need next to phosphate and nitrogen. Sulfur is usually taken up as sulfate. Sulfate uptake and assimilation processes are resembling those known for phosphate and nitrate uptake and assimilation (Kopriva and Rennenberg, 2004; Hesse et al., 2004a). Sulfate is taken up by transport systems in the root and distributed by sulfate transporters in the whole plant (Maruyama-Nakashita et al., 2004; Buchner et al., 2004; Kataoka et al., 2004; Hawkesford, 2003; Hawkesford et al., 2003; Saito, 2000). Excess sulfate is stored in the vacuole while the remainder is reduced to sulfide and incorporated into cysteine. Cysteine is an integral part of proteins determining structure and function of proteins and is involved in redox reactions. Further, cysteine is converted to the nutritionally important amino acid methionine, as well as a wide range of sulfur-containing metabolites, predominant among them glutathione (GSH) and S-adenosylmethionine (SAM) (Hesse et al., 2004b; Hesse and Hoefgen, 2003; Matthews, 1999; Hell and Rennenberg, 1998; Hell, 1997; Miflin and Lea, 1990). The control of cysteine and methionine biosynthesis has been the target of numerous studies at the biochemical and molecular biology level (Riemenschneider et al., 2005; Hesse et al., 2004b; Wirtz et al., 2004; Hell et al., 2002; Nikiforova et al., 2002; Galili and Höfgen, 2002; Berkowitz et al., 2002; Höfgen et al., 2001).

Sulfate availability though does not only influence the biosynthesis of sulfur containing metabolites but also influences other biosynthetic processes in plants (Nikiforova

et al., 2003, 2004, 2005a; Saito, 2004; Hirai et al., 2003; Maruyama-Nakashita et al., 2003; Kutz et al., 2002). Sulfate starvation of plants leads to a series of metabolic and physiological responses aiming at adopting plant metabolism to the available nutrient supply and to acquire a new homeostatic balance (Nikiforova et al., 2005a). These processes are the molecular bases determining agronomical properties such as crop yield and plant health in dependence of sulfate availability (Haneklaus et al., 2003; Galili and Höfgen, 2002; Hesse and Höfgen, 2001; Blake-Kalff et al., 2000).

The primary target site for effects of sulfate deprivation are the sulfur containing metabolites, the amino acids cysteine and methionine and their immediate derived metabolites such as GSH and SAM. Amino acid content in plants is usually balanced in a delicate way (Höfgen et al., 1995). Yet, environmental conditions are affecting plant amino acid compositions. Changes in asparagine and glutamine levels reflect the effectivity of nitrogen assimilation and carbon availability (Lam et al., 1996), while proline levels alter in response to exogenous stress conditions implied on the plant (Kishor et al., 2005; Shinozaki et al., 2003; Rai, 2002). Glutathione concentrations and, thus, biosynthesis of its precursor cysteine are responsive to stress (Noctor et al., 1998), such as cadmium exposure of plants (Heiss et al., 2003; Cobbett et al., 2000a, b).

Carbohydrate derived carbon backbones as well as nitrogen and sulfur contribute to amino acid biosynthesis. Thus, an integration of the respective biosynthetic pathways is necessary to achieve amino acid homeostasis in plants. A number of investigations try to resolve the components and regulatory key points of this concerted regulation (Kopriva and Rennenberg, 2004; Hesse et al., 2004a; Hesse and Hoefgen, 2003). In this study we compile the response of plant amino acid biosynthetic pathways on sulfate depletion of *Arabidopsis thaliana* seedlings, either exposed to starvation on solidified agarose medium, shifting seedlings to 100 μM sulfate instead of about 1 mM under full nutrition (Nikiforova et al., 2003) or in a submerged shaking culture, shifting plants for 2 days from 3 mM sulfate to zero sulfate (M. Bielecka, Max-Planck-Institut für Molekulare Pflanzenphysiologie, Golm, Germany, pers. commun.; Scheible et al., 2004). The plant material has been subdued to a transcriptome and metabolome analysis.

Transcriptome analysis of *Arabidopsis* seedlings in response to sulfur deprivation

Nutrient starvation results in the depletion of endogenous stores of the respective nutrient and of derived organic compounds due to impaired biosynthesis of the respective metabolites. In case of sulfate deprivation, plant tissue sulfate levels, e.g., sulfate stored in the vacuole, total elemental sulfur levels and the levels of the main organic molecules carrying reduced sulfate, i.e., cysteine, glutathione and protein, are reduced (Nikiforova et al., 2003, 2004, 2005a). Homeostasis of amino acid biosynthesis is largely regulated at the level of control of enzyme activities and metabolite fluxes through the pathways with a number of feed back and feed forward mechanisms controlling enzyme activity and leading to accurate pool sizes of free amino acids (Riemenschneider et al., 2005; Hesse et al., 2004b; Hesse and Hoefgen, 2003; Matthews, 1999; Hell and Rennenberg, 1998; Hell, 1997; Miflin and Lea, 1990). Further, degradative processes are involved as well to balance pool sizes (Galili and Höfgen, 2002). When the regulatory properties of the enzymatic machinery cannot cope any longer with the occurring disbalances due to enduring starvation, we assume the induction of further adaptation mechanisms involving alterations in gene expressions. This has been shown in various studies on the expression levels of sulfate transporters and sulfate assimilation pathway genes (Nikiforova et al., 2003, 2004, 2005; Saito, 2004; Hirai et al., 2003; Maruyama-Nakashita et al., 2003; Kutz et al., 2002; Saito, 2000). Recently also a sulfur-responsive *cis*-element in the promoter of some sulfur-responsive genes has been identified (Maruyama-Nakashita et al., 2005). In case of sulfate deprivation of *Arabidopsis* plants in hydroponic cultures, changes in gene expression seem to be triggered about 2 days after onset of starvation (when using artificial transfer of plants to zero or very low levels of sulfate in the root environment) (Nikiforova et al., 2004; B. Gakière, Max-Planck-Institut für Molekulare Pflanzenphysiologie, Golm, Germany, pers. commun.). With continuing starvation the number of induced genes further rises and increasingly involves pathway-unrelated genes (Nikiforova et al., 2004). The accumulation of "downstream" effects is probably caused by cross influencing adjacent pathways due to the lack or accumulation of metabolites and their cumulating effect on correlated physiological processes, eventually resulting in the induction of downstream processes in a snowball-like effect (Nikiforova et al., 2003, 2004, 2005a; Hirai and Saito, 2004). Thus, the analysis of the response at the gene expression level (transcriptome analyses using array technologies) in response to sulfate availability (Table 1) provides insights into the involved mechanisms and responsive pathways (Nikiforova et al., 2003, 2004; Saito, 2004; Hesse et al., 2004a; Hirai et al., 2003; Maruyama-Nakashita et al., 2003; Kutz et al., 2002).

Table 1. Relative ratios reflecting changes in expression of genes involved in amino acid biosynthesis upon sulfate starvation of *Arabidopsis thaliana* seedlings

Biosynthesis and gene	Expression level ratio for seedlings starved for the indicated time and grown in or on:				Average of all 4 experiments
	liquid culture		solidified agarose		
	2 d	2 d	6–10 d	10–13 d	
Arginine biosynthesis:					
At2g48140 carbamoyl-phosphate synthase	4.71	6.71	0.69	0.67	*0.68*
Glutamine biosynthesis:					
At3g17820 glutamine synthetase (GS1)	0.95	2.05	1.94	2.47	*2.20*
At5g37600 glutamine synthetase, putative	1.05	2.25	2.63	2.36	*2.50*
Proline biosynthesis:					
At1g54100 aldehyde dehydrogenase, putative	1.29	2.23	1.58	1.77	*1.67*
Asparagine biosynthesis:					
At3g47340 asparagine synthetase 1	0.70	0.47	1.47	1.01	*1.24*
At4g31990 aspartate transaminase	0.89	0.75	1.88	1.27	*1.57*
Isoleucine biosynthesis:					
At3g19710 branched-chain amino acid aminotransferase (BCAT4)	0.02	0.02	0.71	0.68	*0.69*
Lysine biosynthesis:					
At3g02020 aspartate kinase, lysine-sensitive, putative	0.44	0.43	n.d.	n.d.	*0.43*
At1g31230 bifunctional aspartate kinase/homoserine dehydrogenase	0.58	0.39	n.d.	n.d.	*0.48*
At5g52100 dihydrodipicolinate reductase family protein	0.80	0.45	n.d.	n.d.	*0.63*
At5g46180 ornithine aminotransferase, putative	1.04	1.67	0.54	0.97	*0.76*
Serine and glycine biosynthesis:					
At1g36370 glycine hydroxymethyltransferase, putative	19.85	228.09	5.48	8.46	*6.97*
Cysteine biosynthesis:					
At3g03630 cysteine synthase, chloroplast	0.65	0.10	0.50	0.44	*0.47*
At5g28020 cysteine synthase	2.13	1.46	1.31	1.12	*1.22*
At1g55920 serine acetyltransferase SAT1	2.06	2.92	1.03	1.20	*1.11*
Methionine and SAM biosynthesis:					
At4g23600 cystathionine beta-lyase	2.68	2.24	n.d.	n.d.	*2.46*
At1g64660 cystathionine beta-lyase	2.88	7.13	1.87	1.71	*3.40*
At3g22740 homocysteine S-methyltransferase 3 (HMT-3)	0.09	0.09	n.d.	n.d.	*0.09*
At1g02500 S-adenosylmethionine synthetase 1 (SAM1)	1.00	1.45	1.92	1.28	*1.60*
At3g17390 S-adenosylmethionine synthetase, putative	1.35	1.39	2.38	2.49	*2.44*
Chorismate biosynthesis:					
At2g45300 3-phosphoshikimate 1-carboxyvinyltransferase	n.d.	n.d.	1.45	1.50	*1.48*
At1g48860 3-phosphoshikimate 1-carboxyvinyltransferase	1.12	1.13	2.73	2.50	*2.61*
Phenylalanine biosynthesis:					
At2g24850 aminotransferase, putative	0.12	1.67	0.92	1.48	*1.20*
At1g69370 chorismate mutase, putative	0.86	0.86	0.81	0.70	*0.76*
At5g22630 prephenate dehydratase family protein	1.30	2.18	1.84	1.32	*1.58*
At1g11790 prephenate dehydratase family protein	1.11	0.92	0.55	0.53	*0.54*

(*continued*)

Table 1 (*continued*)

Biosynthesis and gene		Expression level ratio for seedlings starved for the indicated time and grown in or on:				Average of all 4 experiments
		liquid culture		solidified agarose		
		2 d	2 d	6–10 d	10–13 d	
Tryptophan biosynthesis:						
At5g05730	anthranilate synthase, alpha subunit (ASA1)	0.63	0.96	2.90	2.00	*2.45*
At4g30530	GMP synthase (glutamine-hydrolyzing)	1.66	2.14	2.88	2.78	*2.83*
Leucine biosynthesis:						
At5g23020	2-isopropylmalate synthase 2 (IMS2)	0.31	0.25	n.d.	n.d.	*0.28*
At1g31180	3-isopropylmalate dehydrogenase, chloroplast	0.33	0.33	n.d.	n.d.	*0.33*
At3g58990	3-isopropylmalate dehydratase	0.13	0.11	1.16	0.82	*0.99*

Ratios are calculated from expression levels of sulfate-starved to normal sulfate supply levels. Altogether 420 genes and isoforms involved in amino acid biosynthesis were scored for changes under sulfate-deprived growth conditions using *Arabidopsis thaliana* seedlings. Listed are those genes of the plant amino acid biosynthetic pathways which are responding significantly in at least one of the experimental conditions. These 31 genes correspond to about 7.4% of the tested genes. The single columns represent averages of several repetitions each. In column 5 averages were calculated over all experiments to provide a summary of the observed responses. The first pair of data columns represent data from an experiment where seedlings were grown in liquid culture, partially submerged, on agitated shakers (M. Bielecka, pers. commun.). The second pair of data columns represent results for seedlings grown on agarose plates (Nikiforova et al., 2003). Starvation in the first case was two days on zero sulfate; and in the second, several days (6 to 13) on 100 μM sulfate. Sampling for the third data column was performed before the first visible changes caused by starvation could be detected (starvation for 6–10 days); sampling for the fourth column was performed just after identification of the first visible changes such as growth retardation, chlorosis and reddish colour accumulation (starvation for 10–13 days); culture conditions were as described in Nikiforova et al. (2003). Culture conditions for the experiments reported in the first two data columns were as described in Scheible et al. (2004). For the first pair of columns, affymetrix array data were analysed (2 repetitions each), for the second pair of columns, macroarray data using nylon filter hybridisations with spotted ESTs were analysed (10 repetitions each). *n.d.*, not determined. Gene annotation is according to AraCyc from Tair (www.arabidopsis.org)

When concentrating on those genes involved *sensu strictu* in amino acid biosynthesis or the provision of the immediate carbon precursors (Table 1) we can come to following generalisations. First of all, the number of genes showing a significantly altered expression level in at least one of the experimental points is rather low and comprises only about 7% (29 genes) of the 420 tested genes. Further, only few of the genes still exceed a threshold level of factor 2 when average values of several experiments are calculated to compensate for biological variation. Second, the experimental setups with respect to growth conditions and duration of sulfate deprivation (see footnote of Table 1 and Nikiforova et al., 2003; Scheible et al., 2004) lead to a certain extent to a variability in the results. In a few cases such as carbamoyl-phosphate synthase (At2g48140; arginine biosynthesis) and anthranilate synthase (At5g05730; tryptophan biosynthesis) the results appear to be even contradictory.

Despite these considerations, transcriptome analysis still provides some reasonable findings when investigating the general tendencies of being induced or rather reduced under sulfate-limited growth conditions. SHMT (At1g36370) as a central gene in serine–glycine conversion is strongly induced probably in response to the block in cysteine formation which is leading to an accumulation of the cysteine precursor serine. Genes of phenylalanine and tryptophan biosynthesis are induced, while genes of the aspartate branch, to which methionine belongs, are either down regulated as the genes for lysine and isoleucine biosynthesis or seemingly not affected in their expression levels. Yet, genes of the SAM cycle system are induced. As nitrogen assimilation is known to be impaired by S starvation (Hesse et al., 2004a; Kopriva and Rennenberg, 2004), it is of interest to detect that genes of asparagine and glutamine synthesis are induced to increase the sink binding capacity for N when provision of other sinks is impaired by starvation. Generally it has been shown that metabolism is down regulated as protein and RNA levels drop under sulfate-starved growth conditions (Nikiforova et al., 2005a), thus, removing major sinks for nitrogenous organic compounds.

Metabolome analysis of *Arabidopsis* seedlings in response to sulfur deprivation

When the capacities for pools, size regulation by the existing enzyme machinery are exhausted due to depletion of affected precursor pools, the plant is forced into a response cascade resulting in an adjustment of the enzyme composition fitting to the altered environmental conditions. After

sensing disbalances in nutrients or metabolites – in case of S starvation the depletion of pools of reduced sulfur containing organic molecules (cysteine, GSH, and SAM) or sulfate itself and accumulation of the precursors O-acetylserine and serine (Nikiforova et al., 2003, 2005a), alterations in gene expressions are triggered (Table 1). These transcriptional changes need to be converted to changes in enzyme amounts in order to eventually exert an effect on metabolism. The discontinuous response in some cases as well as the overall relatively low number of genes indicates, rather than being discreditable, that the gene response is time dependent and dependent on the respective experimental conditions. Thus, for a complete picture the number of (comparable) experiments is still too low as compared to the variables involved in the system. This provides difficulties for statistical procedures as the number of variables largely exceeds the number of experimental data points. The transcriptional response of the system is eventually transmitted into alterations of metabolite pool sizes and/or

Table 2. Relative ratios of metabolite pools of amino acids and amino acid precursors upon sulfate starvation of *Arabidopsis thaliana* seedlings

Compound	Chemical class	Ratio of metabolite pool for seedlings grown in or on:		
		Liquid culture, 2 days starvation	Solidified agarose, starvation 6–10 days (before visible changes)	Solidified agarose, starvation 10–13 days (after visible changes)
alanine	amino acid	1.99	1.16	2.16
arginine	amino acid	1.01	0.41	0.19
asparagine	amino acid	n.d.	2.90	2.97
aspartate	amino acid	1.77	1.04	0.74
citrulline	amino acid	1.65	n.d.	n.d.
glutamate	amino acid	0.80	1.14	0.97
glutamine	amino acid	1.74	4.15	5.47
glycine	amino acid	5.01	5.77	1.21
homoserine	amino acid	1.98	1.70	1.24
isoleucine	amino acid	1.40	1.89	1.80
leucine	amino acid	1.14	n.d.	n.d.
lysine	amino acid	n.d.	1.34	1.05
methionine	amino acid	0.38	1.12	0.80
O-acetylserine	amino acid	n.d.	16.37	9.09
ornithine	amino acid	n.d.	0.32	0.17
phenylalanine	amino acid	1.49	1.95	1.09
proline	amino acid	0.76	1.06	0.74
serine	amino acid	4.16	2.55	2.12
threonine	amino acid	1.69	1.95	1.41
tryptophan	amino acid	1.45	22.45	6.37
valine	amino acid	1.97	1.99	1.59
allantoin	organic acid	n.d.	10.61	5.17
α-ketoglutarate	organic acid	n.d.	1.64	1.19
citrate	organic acid	n.d.	1.09	0.80
fumarate	organic acid	0.33	1.53	0.97
malate	organic acid	0.30	1.72	0.89
shikimate	organic acid	2.30	0.94	1.17
succinate	organic acid	0.52	1.28	0.74
glucose	sugar	n.d.	1.08	1.32
3-phosphoglycerate	sugar alcohol	n.d.	3.43	3.42
pyruvate	organic acid	1.04	n.d.	n.d.
SAM (AdoMet)	nucleoside	n.d.	0.22	0.03

Ratios are presented as sulfate starved to control plants. Plant metabolites were determined by GC-MS; SAM was determined by LC-MS (Nikiforova et al., 2005a). Relative values of abundance were obtained in comparison to ribitol as internal standard. The same material was used as for transcriptome analysis (see footnote of Table 1). Listed are all amino acids and metabolites involved in providing the carbon backbones for amino acid biosynthesis. In the latter case only those metabolites are depicted which are exhibiting significant alterations in at least one of the experimental conditions. SAM has been determined by LC-MS and represents the major downstream sink of methionine biosynthesis and a central metabolite in plant C1 metabolism. However, it has to be noted that the amount of nonannotated peaks is about 57% for GC-MS analysis at the current state (Nikiforova et al., 2005a). Sampling was as described for Table 1; the first data column of this table corresponds to the second data column of Table 1. *n.d.*, not determined

metabolite fluxes. These alterations are corresponding to an adjustment of the metabolite pool sizes and a rebalancing of the resources in adaptation to a nutrient limitation. Hence, metabolite pool sizes represent the integration of disturbed biosynthetic pathways, altered gene expression levels and altered enzyme abundancies and activities and are thus a good indicator for the response of the entire system.

Metabolite pools in this study have been determined by GC-MS or LC-MS based metabolite profiling (Nikiforova et al., 2005a; Fiehn et al., 2000). Contents of plant amino acids and some of their precursors in sulfate-deprived plants (Table 2) reveal a differentiated response and not a uniform alteration of all amino acids. The reduced sulfate availability provides a block for cysteine synthesis as insufficient amounts of sulfide are provided through the uptake and sulfate reduction pathway. This leads in a classical way to a reduction of the immediate products, cysteine and glutathione, while the precursors, O-acetylserine and serine, accumulate (Nikiforova et al., 2005a). As serine is linked closely to glycine formation (Li et al., 2003; Bauwe and Kolukisaoglu, 2003), the concurrent accumulation of glycine is following expectations. Cysteine itself serves as precursor of methionine through a transsulfuration reaction (Hesse et al., 2004b; Hesse and Hoefgen, 2003). Unexpectedly though, methionine levels are not grossly affected but kept relatively constant over a wide time range of sulfate deprivation (Nikiforova et al., 2005a) or slowly decrease under zero sulfate (M. Bielecka, pers. commun.). Thus, despite the almost complete depletion of cysteine (Nikiforova et al., 2003), the plant is able to keep methionine levels, which are always low in plants, constant. This indicates the indispensability of this metabolite as has been shown in various studies. The main endpoints of methionine synthesis are on the one hand incorporation into proteins and on the other hand the biosynthesis of the main plant C1-metabolism methyl donor, S-adenosylmethionine (SAM). SAM is involved in numerous methylation reactions but also serves as precursor of methionine, ethylene, and polyamine biosynthesis and – in some plants – S-methylmethionine biosynthesis, a potential transport moiety of reduced sulfur. Usually, SAM is recycled and resynthesized after its involvement in the respective reactions. Hence, the reduction of SAM pool sizes in S-starved plants will be responsible for various downstream effects and physiological changes.

The carbon backbone of methionine is derived from oxaloacetate and aspartate, placing this amino acid in the aspartate family of amino acids. Other aspartate family members such as threonine and isoleucine 'profit' from the seemingly reduced flow to methionine and SAM as they are both increased in their contents. Lysine levels remain constant. Interestingly, homoserine, a precursor of both threonine and isoleucine biosynthesis and methionine biosynthesis accumulates, indicating a demand for precursors of methionine biosynthesis probably due to the demand for methionine and SAM synthesis. (Lee et al., 2005; Kreft et al., 2003; Zeh et al., 2002; Hesse et al., 2001).

The pyruvate-derived amino acids alanine and valine increase in content, leucine level remains constant, at least in one of the experimental setups.

Sulfur-deprived plants show an accumulation of phenolic compounds as is typical for all nutrient starvations and various other environmental stresses such as cold, drought or high light and easily observable through changes of the leaf colour (Nikiforova et al., 2003, 2004, 2005; Hirai and Saito, 2004; Hirai et al., 2003). These phenolic compounds, e.g., anthocyanin, are derived from the aromatic amino acids tyrosine, phenylalanine and tryptophan. Under sulfur stress we observe a slight increase of the common precursor shikimate, further tryptophan accumulates even to high levels in certain experimental conditions (Table 2) while phenylalanine slightly increases. Tyrosine could not be determined through GC-MS. As serine is directly involved in tryptophan biosynthesis, the accumulating serine under sulfur-deficient growth conditions might well be utilized for the increase of tryptophan contents. The observed accumulation of the aromatic amino acids is in concordance with the accumulation of their downstream, secondary products. Thus, we can assume that the flux through this pathway might even be higher than indicated by the observed increase of metabolites.

While sulfate assimilation is impaired, nitrogen assimilation continues, though cross influences have been described (Hesse et al., 2004a; Kopriva and Rennenberg, 2004). The relative ratio of N to S is still shifted towards an excess of N. Reduced nitrogen is bound to asparagine and glutamine, bearing a second amino group making them suitable for transport of reduced nitrogen and as a substrate for transamination reactions. Both metabolites are increased in their amounts, seemingly buffering an excess of reduced nitrogen under sulfur-limiting conditions. The seemingly low ratio of increase (about 3 times for asparagine and 4 to 5 times for glutamine) might not be misinterpreted. The molar amounts of asparagine and glutamine are usually much higher than those of all other

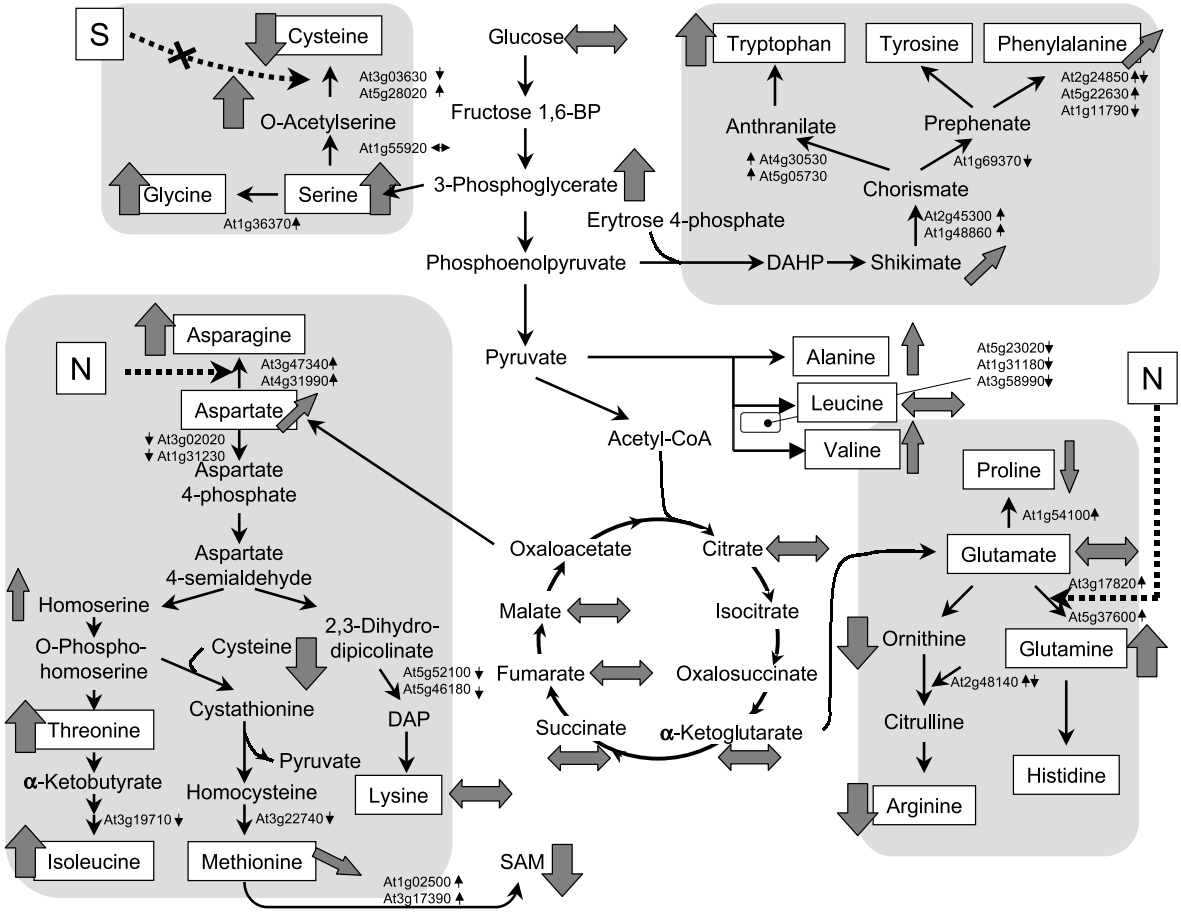

Fig. 1. Superposition of changes in metabolite pools and gene expression on the amino acid biosynthetic pathways and the main anaplerotic reactions providing the carbon backbone precursors of plant amino acids of *Arabidopsis thaliana* seedlings exposed to sulfate deficiency. The amino acids are grouped according to the biosynthetic families (Buchanan et al., 2000). Average values of several independent experiments have been calculated. Grey arrows indicate changes in metabolites, thickness of the arrows provides an indication on the strength of the observed changes and direction of the arrow indicates induction (up), reduction (down) or no alteration (horizontal) of the respective metabolite. Gene names are provided as Atg codes following AraCyc nomenclature (TAIR; www.arabidopsis.org). Only those genes were depicted for which changes were detected using array technology. Arrows indicate induction (up) or reduction (down) under sulfate starvation compared to controls

amino acids, thus, such an increase provides a huge sink for carbon and nitrogen. Despite this obvious tendency to store excess nitrogen in N-rich amino acids the non-protein amino acids ornithine and arginine are rather reduced in content.

Extending this analysis to the precursors of the various amino acid family branches (Fig. 1), we only see marginal alterations of, e.g., TCA cycle metabolites. With respect to carbohydrate backbone supply via glycolysis we only observed an increase of 3-phosphoglycerate while the levels of others remained constant.

Merging expressional and metabolite data

When mapping the transcript and metabolite data (Tables 1 and 2) on the known amino acid biosynthetic pathways (Buchanan et al., 2000), a consistent picture of responses starts to emerge (Fig. 1). Despite the distinct challenge of the system by deprivation of sulfate we observe a rather complex response with cross-effects on other pathways. Compilation of various analyses such as GC-MS and LC-MS based determination of metabolites (Nikiforova et al., 2005a; Fiehn et al., 2000) and unbiased screenings using array technologies (Nikiforova et al., 2003, 2005b) provides a first description of the system response. Some of the results are obviously consistent with the expectations or corroborate previous findings, such as *SAT* induction and OAS accumulation and serine/glycine accumulation coupled to serine hydroxymethyltransferase (SHMT) induction. However, even more far reaching explanations appear possible. Folates are refueling the SAM-C1 transfer cycle through methylation of homocys-

teine to methionine (Zeh et al., 2002; Hesse et al., 2001). As SAM levels are dropping accompanied by a reduction in at least one isoform of the methionine synthase (Table 1), folates might accumulate and might be speculated to have a feedback effect on their own synthesis, again making the accumulation of the folate precursors serine and glycine likely. The cysteine depletion as well results in an induction of *OASTL*. Further, S starvation resulting in SAM depletion induces genes of SAM synthesis and recycling to reconvert the demethylated SAM back to methionine and, eventually, SAM. Astonishingly the methionine levels, though, remain constant under certain experimental conditions (Nikiforova et al., 2005a). First, free methionine concentrations are commonly low in plant tissues but balanced through a complex regulatory system mainly acting at the branch point of the threonine–methionine diversion (Kreft et al., 2003). Threonine synthase (TS) activities are induced by accumulating SAM levels, which are missing here, while SAM or another methionine-derived metabolite regulates additionally the transcript stability and thus abundance of CgS negatively in *Arabidopsis thaliana* (Hesse and Hoefgen, 2003). Still, threonine and isoleucine accumulate under these conditions, mimicking the overproduction of TS through over expressing a TS gene (Muhitch, 1997). An indication though of a demand-driven alteration in upstream processes is given by the accumulation of homoserine, and as can be speculated, probably O-phosphohomoserine, the common substrate for both, methionine and threonine synthesis (Lee et al., 2005; Hesse and Hoefgen, 2003; Kreft et al., 2003). While through the depletion of cysteine, methionine synthesis is impaired, threonine and isoleucine obviously accumulate despite the missing activation of TS through SAM. Again this is a proof for the critical regulation of threonine/methionine homeostasis through the CgS/TS branch point. Only when the branch point enzymes are altered, the plant responds with alterations in methionine levels as shown through the analysis of mutants altered in CgS or TS activity or transgenic approaches altering CgS and TS expression and hence enzyme abundance (Chiba et al., 1999, 2003; Kreft et al., 2003; Bartlem et al., 2000). Manipulation of downstream genes of methionine synthesis, CbL and MS, has no effect on methionine levels (Zeh et al., 2001, 2002; Maimann et al., 2000, 2001). Reduced protein synthesis or even protein breakdown under conditions of sulfate starvation might help to keep methionine levels constant (Nikiforova 2005a) through reducing the loss of methionine into proteins to keep C1-metabolism functional even under conditions of severe sulfate starvation.

The pyruvate-derived amino acids, alanine and valine, are increased about twofold in content. Valine and leucine essentially use the same biosynthetic pathway enzymes as isoleucine, which is increasing also about twofold. The pathways only differ in the precursors used for biosynthesis. Leucine and valine apply pyruvate as substrate while isoleucine derives from deaminated threonine, i.e., 2-oxobutyrate. Thus, this increase seems to be coupled. No alteration in gene expression, though, of the branched chain amino acids could be detected, besides decreases of the genes of the leucine biosynthetic branch. We thus must assume rather a regulation at the enzyme activity and control level. Direct amination of pyruvate leads to alanine which might thus provide another additional N dump and explain the accumulation of alanine. The accumulation of 3-PGA might potentially be an effect of the serine-glycine accumulation.

Other responses affecting amino acid composition are not immediately conclusive. Among the aromatic amino acids the precursor shikimate and phenylalanine show a slight increase while tryptophan shows clearly elevated levels. Some of the genes of phenylalanine biosynthesis as well as genes of shikimate and tryptophan biosynthesis are induced supporting the metabolite data. Downstream metabolites of the phenylpropanoid pathway derived from aromatic acid precursors show also elevated levels in sulfate-stressed plants (Nikiforova et al., 2005a; Hirai and Saito, 2004). Especially, the tryptophan-derived anthocyanins are accumulating (Nikiforova et al., 2003). To which extent this ''stress-related'' response of accumulation of aromatic compounds is sulfate specific at certain regulatory points or is just following a general stress-induced pathway needs to be still elucidated. The fact as such is well known as many plants show reddish colours of the leaf canopy when exposed to stress conditions, be it cold, drought, or nutrient deprivations of either N, P, or S. This adaptation might be of extreme importance under sulfate starvation as glutathione levels are reduced upon S starvation, thus affecting the ascorbate-glutathione redox system (Noctor et al., 1998). We postulate that accumulation of aromatic compounds is triggered to compensate for the loss of this scavenging system of reactive oxygen species and its substitution by alternatively synthesized phenolic compounds. Interestingly, while serine cannot be converted to cysteine and eventually glutathione any longer, it might immediately serve as precursor in tryptophan biosynthesis from indole and serine. Notably, among the most highly induced genes all studies identified a putative isoflavonoid reductase homologue (At1g75280) (Nikiforova et al., 2003; Hirai et al., 2003).

One major problem for plants exposed to sulfate starvation results from the necessity to deal with the relative excess of nitrate and ammonium, which is derived as well from protein and amino acid degradation as from uptake and assimilation. Carbohydrate and organic acid levels are remaining more or less constant under sulfate-deprived conditions, even though one of the effects of sulfate depletion is the reduction of chlorophyll contents probably due to the reduced SAM levels necessary for methylation steps during chlorophyll biosynthesis. Chlorosis is a clear indicator for sulfate starvation, also under agronomical conditions. The imbalance of ''normal'' carbon backbone supply and ''normal'' nitrate assimilation while certain amino acids are impaired in synthesis (cysteine) asks for an integration of carbon and nitrogen metabolism with sulfate metabolism (Hesse et al., 2004a; Kopriva and Rennenberg, 2004). As well, genes as metabolites of the major nitrogen-transporting amino acids, glutamine and asparagine, are increased to compensate the shift in balance of N/S towards N and to prevent ammonia intoxication of the plant. Excess reduced nitrogen seems to be further channeled to ureides (allantoin), derived from purin degradation via xanthin (Nikiforova et al., 2005a). A process also known from, e.g., leguminosous plants such as soy beans which face a similar problem of relative N excess when fixing nitrogen in bacteroids and exporting it to the plant. N-rich ureides though being costly in synthesis are used as major N transport form, while others, such as peas, mainly transport asparagines and glutamine (Lam et al., 1997; Serraj et al., 2001; Khadri et al., 2001).

Conclusions

At low sulfate supply levels this shift in balance and regulation of amino acid biosynthesis allows the plant to readjust its homeostasis and to remain viable and produce seeds for dispersal. The integrity of the amino acid biosynthesis system is kept, though shifted. In case of continued starvation, such as the artificial zero sulfate supply situation imposed in experimental conditions, disturbances accumulate and propagate through the system by triggering further downstream reactions leading finally to plant death when vital components fail to be synthesized at all.

The response of plant metabolism to decreased sulfate availability is governed, at the amino acid biosynthetic level, by three main processes. First, the lack of sulfate and thus reduced sulfide provision leads to a halt in cysteine biosynthesis and its downstream sulfur-containing derivatives such as glutathione and S-adenosylmethionine and an accumulation of precursors of cysteine synthesis, O-acetylserine, serine and glycine, and maybe 3-PGA. These metabolic changes obviously impair already a number of physiological processes, forcing the plant to shift to alternative strategies to remain viable. Second, continued carbon backbone provision and nitrate assimilation coupled to reductions in protein synthesis and further biosynthetic processes such as a damped C1-metabolism and reduced chlorophyll and lipid biosynthesis (Nikiforova et al., 2005a) lead to a relative imbalance of nitrogen over sulfur contents. Excess nitrogen then triggers processes to dump reduced nitrogen into various N-rich sink molecules as glutamine, asparagine and ureides. It can be speculated that this strategy helps to eventually prevent ammonia intoxication. Third, a lack of cysteine leads to reduced glutathione (GSH) levels and a disturbance of the central cellular active oxygen scavenging system, the GSH-ascorbate cycle. Probably in order to deal with this threat of a general weakening of the ability of plants to deal with stresses (Riemenschneider et al., 2005; Bloem et al., 2004; Haneklaus et al., 2003) the biosynthesis of aromatic secondary compounds is induced which might functionally substitute the GSH-ascorbate cycle.

In agricultural practice even moderate sulfate starvations lead to effects on yield and plant performance such as stress and pathogen resistance, or more generally, insufficient sulfate availability impairs the ability of plants to cope with additional stresses. Severe insufficiencies lead to acute growth and yield depressions (Haneklaus et al., 2003; Blake-Kalff et al., 2000).

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